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Cell-mediated immune responses to a killed *Salmonella enteritidis* vaccine: lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-γ production

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Abstract

Two experimental approaches were used to investigate the immunological responses of chickens to a commercial killed *Salmonella enteritidis* (SE) vaccine. In the first, the effects of host age on antigen-specific proliferative responses and cytokine production were examined. Compared with non-vaccinated controls, 4-wk-old vaccinated chickens showed higher proliferation to SE LPS and flagella. The lymphoproliferation responses to these antigens of 8-mo-old vaccinated chickens were not different compared to the non-vaccinated controls. Increased production of interferon-γ (IFN-γ) and interleukin-2 (IL-2) by antigen-stimulated splenocytes following vaccination were, in general, more often observed in 4-wk-old compared with 8-mo-old chickens, whereas serum levels of these cytokines were consistently higher in the vaccinated birds compared with controls regardless of age. The second set of experiments were designed to determine the effects of SE vaccination on

Abbreviations: SE, *Salmonella enteritidis*; ppi, post-primary immunization; psi, post-secondary immunization; OMP, outer membrane protein; HK-SE, heat-killed SE; HBSS, Hanks' balanced salt solution; RPMI-10, RPMI-1640 medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin; FCS, fetal calf serum; SI, stimulation index; PBS-T, PBS containing 0.05% Tween; DTH, delayed-type hypersensitivity; WST-8, 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt

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mitogen- or antigen-induced splenocyte proliferation and serum nitric oxide (NO) and cytokine levels. Splenocytes from vaccinated chickens stimulated with SE flagella showed significantly increased numbers of TCR$\gamma\delta^+$ cells at 7 days post-vaccination compared with non-vaccinated birds. In contrast, no differences were noted with $CD4^+$, $CD8^+$, or TCR$\alpha\beta^+$ cells at any time points examined. Higher levels of NO production were observed following stimulation with SE flagella at 4, 7, 11, and 14 days after SE vaccination while serum levels of IFN-γ, IL-1, IL-6, and IL-8 were elevated only at day 7 post-vaccination. In conclusion, younger chickens mounted a more robust antigen-specific immune response to the SE vaccine compared with older birds and vaccination induced not only T-cell-mediated responses but also host innate and pro-inflammatory responses.
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Keywords: Chicken; *Salmonella enteritidis*; Killed vaccine; Cell proliferation; Cytokines

Résumé

Deux approches expérimentales ont été utilisées pour examiner les réactions immunologiques de poulets au vaccin tué commercial *Salmonella enteritidis*. La première visait à observer les effets de l'âge du sujet sur les réactions prolifératives spécifiques aux antigènes et la production de cytokine. Par comparaison avec des témoins non vaccinés, les poulets de 4 semaines vaccinés ont montré un taux supérieur de prolifération de LPS et flagelles de SE. Les réactions de lymphoprolifération à ces antigènes chez des poulets vaccinés âgés de 8 mois n'étaient pas différentes de celles de témoins non vaccinés. On a en général observé l'augmentation de la production d'interférone-γ (IFN-γ) et d'interleukine-2 (IL-2) par des splénocytes stimulés par des antigènes à la suite de la vaccination plus souvent chez des poulets de 4 semaines que chez des poulets de 8 mois, tandis que les niveaux de sérum de ces cytokines étaient régulièrement plus élevés chez les volatiles vaccinés que chez les témoins non vaccinés, quel que soit leur âge.

La seconde série d'expériences était conçue pour déterminer les effets de la vaccination SE sur la prolifération de splénocytes causée par des mitogènes ou des antigènes, et sur les niveaux d'oxyde nitrique (NO) et de cytokine. Les splénocytes de poulets vaccinés stimulés avec flagelles SE montraient des taux de cellules TCR$\gamma\delta^+$ beaucoup plus élevés à 7 jours après la vaccination que ceux de volatiles non vaccinés. Des taux de production de NO supérieurs étaient observables à la suite d'une stimulation avec flagelle SE à 4, 7, 11 et 14 jours après vaccination SE tandis que les niveaux de IFN-γ, IL-1, IL-6, et IL-8 n'étaient supérieurs qu'au 7ème jour après la vaccination. En conclusion, des poulets plus jeunes ont témoigné d'une réponse immunitaire spécifique aux antigènes au vaccin SE plus forte que celle de volatiles plus âgés, et la vaccination a produit non seulement des réponses médiatisées par les cellules T mais aussi des réponses innées et pro-inflammatoires chez les sujets.
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Mots-clé: Poulet; *Salmonella enteritidis*; Vaccin tué; Prolifération des cellules; Cytokines

1. Introduction

Salmonellosis is one of the most important food-borne zoonotic disease throughout the world and poultry represents an important source of infection in man. The incidence of food poisoning due to consumption of *Salmonella enteritidis* (SE)-contaminated eggs is

still a major concern for food safety worldwide [1–5]. Both horizontal SE infection between adult birds and vertical transmission from the ovary and/or oviduct of laying hens to their eggs are responsible for human *Salmonella* outbreaks [6–11]. One hypothesis to account for vertical SE infection relates to differential immunological and inflammatory responses between immature and adult chickens [12]. Several studies have clearly documented that susceptibility of chickens to SE is age-dependent, possibly related to the maturation status of the host immune system. While adult egg-layers produced SE-contaminated eggs at low frequency in the absence of overt clinical symptoms and viable bacteria were isolated from their internal organs [6,13–15], immature chicks exposed to SE often showed clinical signs of infection [16]. In addition, 2-wk-old chickens infected with SE had viable bacteria in their intestinal ceca, whereas no organisms were detected in adult birds following an identical bacterial exposure [17]. It is important to note, however, that results contradictory to these also have been published. Thus, hens at 20 wk of age were less susceptible to SE infection than those at 55 wk of age [14] and hens infected orally with SE at 62 wk of age were more often colonized in their intestine and produced more SE-contaminated eggs compared with chickens at 27 or 37 wk [18].

A number of different strategies have been attempted to control SE infection in chicken flocks with the intent of protecting humans from SE food poisoning. Among the most promising of these are development and use of *Salmonella* vaccines. Several studies have suggested that the live attenuated vaccines are more effective than killed vaccines in preventing SE infection [19]. However, further studies on the efficacy of killed vaccines are needed because of potential hazards associated with the use of live attenuated SE strains, such as reversion to virulence [20] and immunosuppression [21–24]. Because of these concerns, we undertook the present study to evaluate several parameters of innate and acquired host immunity in response to vaccination of chickens with a commercial killed SE vaccine. The data suggest that the SE vaccine used under specific conditions may stimulate an appropriate host immune response in commercial poultry flocks.

2. Materials and methods

2.1. Animals and immunizations

Specific pathogen-free White Leghorn SC inbred chickens (Hy-vac Adel, IA) were obtained as fertile eggs, hatched at the Animal and Natural Resources Institute, maintained in brooder batteries until 3 wk of age, transferred to floor pens, and provided with commercial broiler ration and water ad libitum. The study was performed according to the Beltsville Agriculture Research Center Small Animal Care Committee guidelines. In experimental design #1, 18 chickens each at 4 wk or 8 mo of age were vaccinated intramuscularly in the leg with a commercially available killed SE vaccine (Poulvac® SE, Fort Dodge Animal Health, Overland Park, KS) at an inoculum of 0.2 (for 4-wk-old) or 0.4 (for 8-mo-old) ml per chicken, respectively. Fifteen days post-primary immunization (ppi), chickens in both groups were immunized again with the same vaccine dose they received previously. Non-vaccinated chickens in both age groups were used as negative controls. At 4, 7, 11, and 14 days ppi and 3 and 6 days post-secondary immunization (psi),

three chickens from each group sacrificed for collection of sera and spleens. Splenocytes were isolated immediately as described below and sera stored at −20 °C until use. In experimental design #2, 36 chickens were vaccinated intramuscularly in the leg (0.3 ml/chicken) at 3 wk of age with the Poulvac® SE vaccine. Fifteen days ppi, chickens in the vaccinated groups were injected again with the same vaccine dose they received previously. Another group of unvaccinated chickens was used for the control. At 4, 7, 11, and 14 days ppi and 3 and 6 days psi, six chickens from each group were bled by cardiac puncture for serum collection and their spleens were obtained for examinations described below.

2.2. Preparation of antigens

Lipopolysaccharide (LPS, Difco, Detroit, MI), outer membrane protein (OMP), porin, and flagella were prepared from SE strain FDA338 from bacteria grown at 37 °C overnight in Trypticase soy broth (Difco, Detroit, MI) supplemented with yeast extract. The bacteria were washed two times with PBS by centrifugation at 4500*g* for 30 min at 4 °C and adjusted to approximately 10^9 cfu/ml in PBS. To prepare OMP, bacteria were sonicated three times for 1 min on ice, followed by centrifugation at 1500*g* for 30 min at 4 °C. The supernatant was centrifuged at 20,000*g* for 30 min, and OMP was obtained as the pellet. Porin was obtained as the clear gel-like pellet by centrifugation at 20,000*g* after dissolving OMP with 2% (wt/vol) sodium dodecyl sulfate. For preparation of flagella, bacteria were homogenized in PBS with a tissue demembranator (OMNI International, Warrenton, VA) at 30,000 rpm two times for 30 s and flagella obtained from the supernatant by centrifugation at 2000*g* for 30 min. Protein concentrations were determined by the method of Lowry et al. [25]. Heat-killed (HK) SE was prepared by boiling bacteria at 100 °C for 10 min.

2.3. Mitogen- and antigen-induced lymphocyte proliferation assays

Spleens were teased through the cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) into a 100 mm petri dish containing Hanks' balanced salt solution (HBSS), the cell suspension overlaid onto Histopaque 1077® (Sigma, St Louis, MO) and centrifuged at 400*g* for 20 min at room temperature. Lymphocytes at the interface were collected, washed twice with HBSS at 250*g* for 5 min at 4 °C, and resuspended in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St Louis, MO) (RPMI-10). More than 95% of cells were determined viable by trypan blue dye exclusion. Spleen cells (1.25×10^6 per well) were seeded into the 96-well tissue culture plates and stimulated with concanavalin A (ConA, 12.5 μg/ml), or SE OMP (2.0 μg/ml), porin (3.0 μg/ml), flagella (2.0 μg/ml), or HK-SE (10^5 CFU/ml) for 48 h at 41.5 °C and 5% CO_2. 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt (WST-8, Cell-Counting Kit-8® (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) 10 μl per well) was added as described [26], the plates incubated for 4 h, and the optical density (OD) measured at 450 nm using a microplate reader (BioRad, Hercules, CA). Each sample was analyzed in six replicates and the stimulation index (SI)

calculated using the following formula: SI = (mean OD of mitogen- or antigen-stimulated proliferation)/(mean OD of non-stimulated proliferation).

2.4. Enzyme linked immunosorbent assay

Spleen cells were stimulated with ConA or *Salmonella* antigens as described above in 24-well tissue culture plates for 48 h, culture supernatants collected, filtered through 0.45 um membrane (Schleicher and Schuell, Inc., Keene, NH), and frozen at − 20 °C until use. 96-well ELISA plates were coated in quadruplicate with 100 μl per well of serum or culture supernatant in carbonate–bicarbonate buffer as described [27] and the plates incubated for 20 min at room temperature and 4 °C overnight. After washing six times with PBS containing 0.05% Tween (PBS-T), 100 μl per well of 1% BSA-PBS was added, the plates incubated for 1 h at room temperature, the liquid decanted, and 50 μl per well of a predetermined dose of monoclonal antibody (mAb) against chicken IL-1, IL-2, IL-6, IL-8, or interferon-γ (IFN-γ) [27–29] added. The plates were incubated for 1 h at room temperature, washed five times with PBS-T, 100 μl per well of peroxidase conjugated goat anti-mouse IgG antibody (Sigma, St Louis, MO) (1:2000) in 0.1% BSA-PBS added, incubated for 1 h at room temperature, washed, and 100 μl per well of peroxidase substrate (Sigma, St Louis, MO) added. After 5 min incubation, substrate reaction was stopped with 50 μl per well of 2N H_2SO_4, and the OD values measured at 450 nm (BioRad, Hercules, CA). Each assay was performed in triplicate. Recombinant cytokines and vector alone were used as positive and negative controls, respectively.

2.5. Flow cytometric analysis

Splenocytes were washed two times and resuspended in 1.0 ml HBSS containing 3% FCS and 0.01% sodium azide. One hundred μl aliquots of cell suspensions (approximately 10^6 cells) were incubated on ice for 40 min with 100 μl of appropriately diluted mAb against CD4, CD8, TCRαβ, or TCRγδ [30,31]. MAbs against HB2 (ATCC, Manassas, VA) (human T-cell marker) and K55 (pan chicken lymphocyte marker) were used for the negative and positive controls, respectively. After washing two times, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma, St Louis, MO) for 30 min on ice, the cells washed two times, resuspended in 2.0 ml, and analyzed with a Epics model XL flow cytometer (Coulter, Miami, FL). Data were obtained from a total of 10^4 viable cells in duplicate.

2.6. NO assay

Thawed cell culture supernatants were centrifuged at 200*g* for 5 min at 4 °C, 100 μl were mixed with 100 μl of freshly prepared Griess reagent (Sigma, St Louis, MO) in flat-bottom 96-well plates, the plates incubated for 15 min at room temperature, and the OD measured at 540 nm. Nitrite concentration was determined using a standard curve generated with sodium nitrite. Each sample was assayed in six replicates.

2.7. Statistical analysis

Differences between groups were compared by the Student's *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA) and considered significant at $P < 0.05$.

3. Results

3.1. Effects of vaccination age on ConA or SE antigen-specific lymphocyte proliferation

The first set of experiments were designed to test the effects of age on lymphocyte proliferation and cytokine production following vaccination with the Poulvac® killed SE

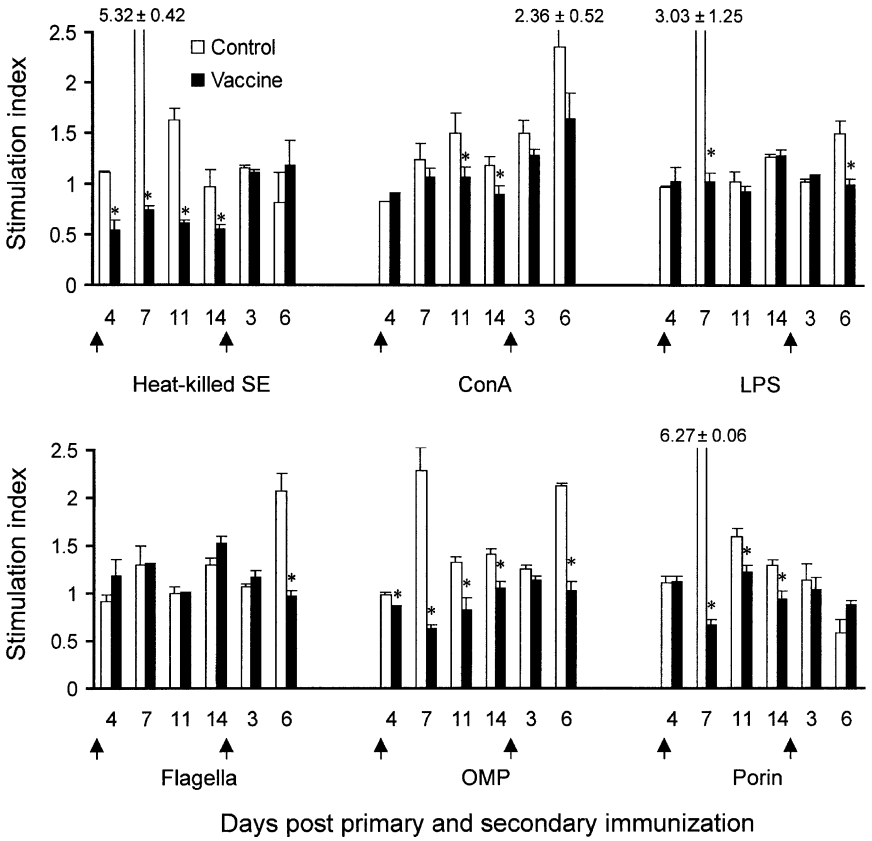

Fig. 1. Mitogen- and SE antigen-induced proliferation of splenocytes from chickens vaccinated at 8 mo of age. Arrows indicate primary (day 0) and secondary (day 15) vaccinations. At 4, 7, 11, and 14 days ppi, and 3 and 6 days psi, splenocytes were collected and cultured in the presence of ConA or SE antigens for 48 h. Cell proliferation was measured by the WST-8 assay as described in Section 2. The stimulation index (SI) was calculated by the following formula: SI = (mean OD of mitogen- or antigen-stimulated proliferation)/(mean OD of non-stimulated proliferation). Each bar represents the mean SI ± SD of three birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$). SI values >2.5 are listed numerically.

vaccine. Splenocytes from chickens vaccinated at 8 mo of age either showed similar (ConA, LPS, or flagella) or decreased (HK-SE, OMP, or porin) proliferative responses compared with age-matched non-vaccinated birds (Fig. 1). While chickens vaccinated at 4 wk of age were similar to the 8-mo-old group in that a similar response to HK-SE and porin were observed, several differences between the two age groups were also apparent (Fig. 2). For example, splenocyte proliferation in response to LPS or flagella in 4-wk-old chickens was consistently and significantly higher in vaccinated animals compared with non-vaccinated controls. Secondly, a greater response was observed in vaccinated chickens vs. non-vaccinated controls in splenocytes stimulated with ConA or OMP at 4 days ppi ($P < 0.05$).

3.2. Effects of vaccination age on IFN-γ and IL-2 production by SE-immune splenocytes

Splenocytes from chickens vaccinated at 8-mo-old showed no significant differences compared with non-vaccinated chickens in IFN-γ production after in

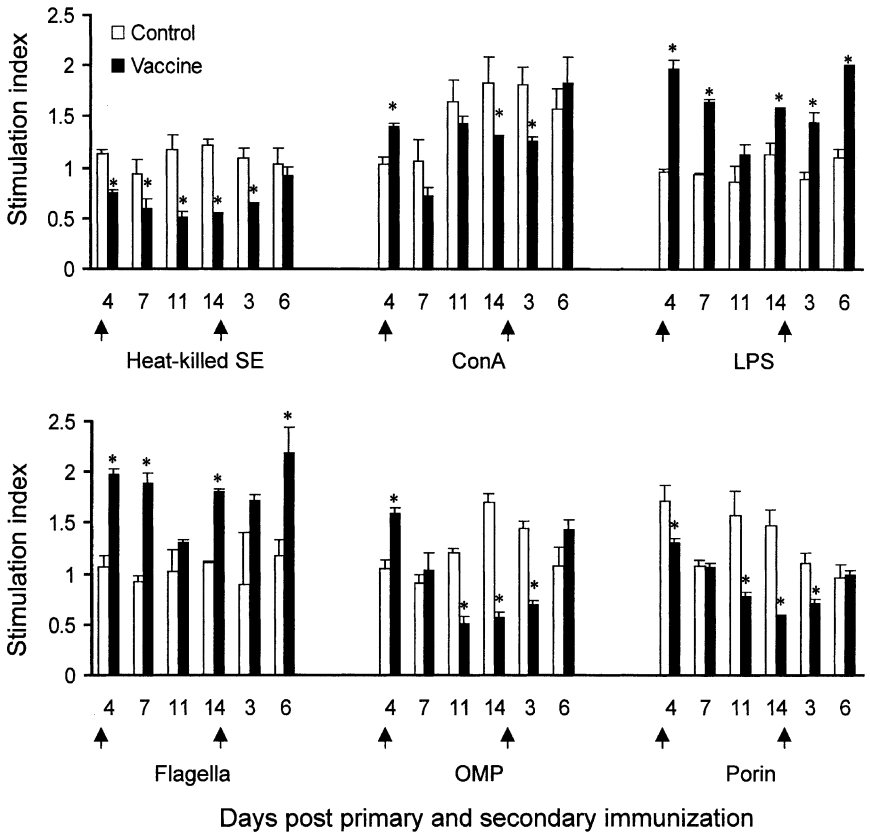

Fig. 2. Mitogen- and SE antigen-induced proliferation of splenocytes from the chickens vaccinated at 4 wk of age as described in Fig. 1.

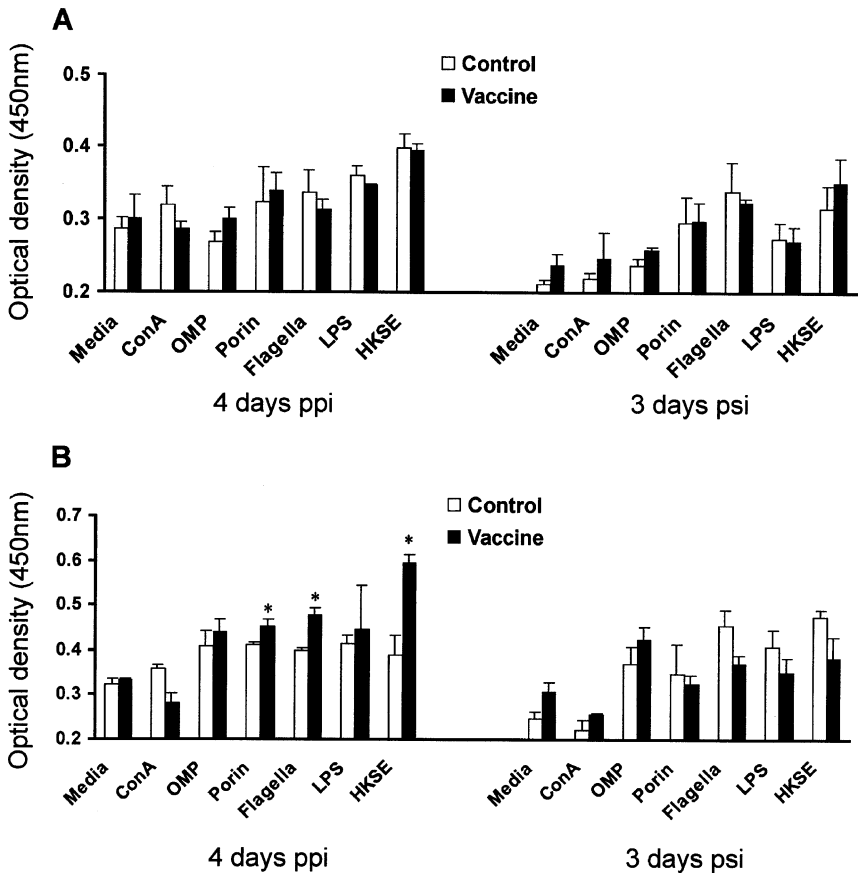

Fig. 3. Mitogen- and SE antigen-induced IFN-γ and IL-2 production by splenocytes from the chickens vaccinated at 8 mo of age. At day 4 ppi and day 3 psi, splenocytes were collected from chickens and cultured with ConA or SE antigens for 48 h. IFN-γ and IL-2 in the culture supernatants were measured by ELISA as described in Section 2. Each bar represents the mean ± SD of three birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$).

vitro stimulation for 4 days ppi or 3 days psi with ConA or any of the SE antigens tested (Fig. 3A). In contrast, significantly higher levels of IL-2 production were observed in 8-mo-old vaccinated chickens following stimulation with porin, flagella, or HE-SE at 4 days ppi compared with the non-vaccinated group (Fig. 3B). Interestingly, this effect was not seen following secondary immunization. Chickens vaccinated at 4 wk of age, however, showed significantly increased IFN-γ production by splenocytes following stimulation with ConA, OMP, porin, or flagella at 4 days ppi or 3 days psi (Fig. 4A). Similarly, splenocytes of chickens vaccinated at 4 wk produced significantly higher IL-2 following stimulation with flagella, LPS, or HK-SE at 4 days ppi or with ConA, OMP, porin, or flagella at 3 days psi compared to the age-matched controls (Fig. 4B).

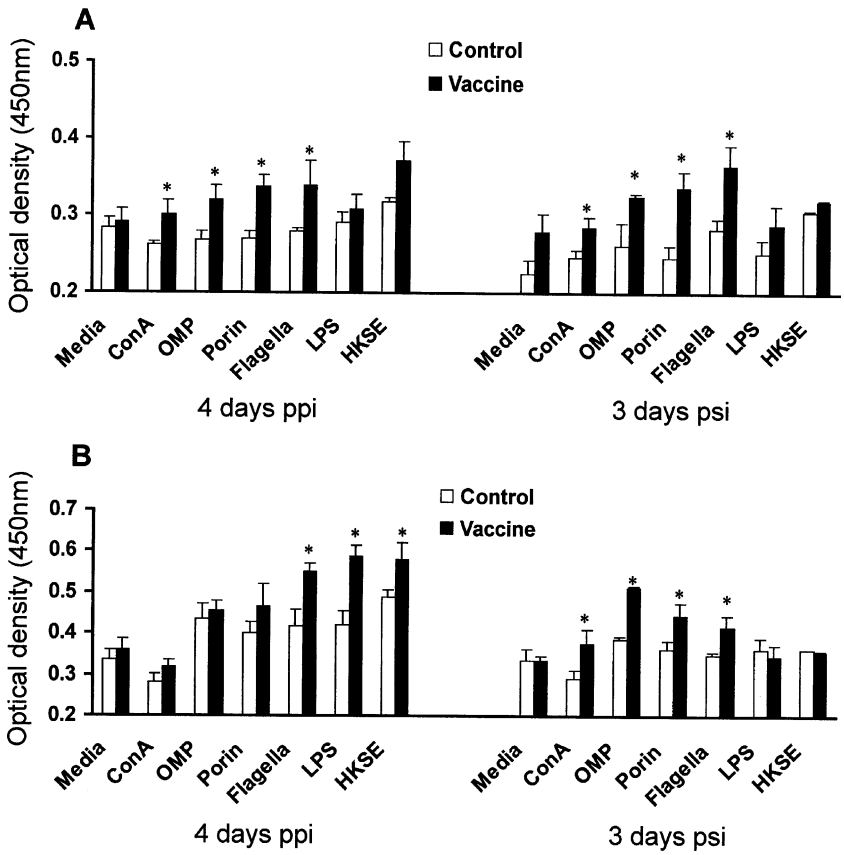

Fig. 4. Mitogen- and SE antigen-induced IFN-γ and IL-2 production by splenocytes from the chickens vaccinated at 4 wk of age as described in Fig. 3.

3.3. Effects of vaccination age on serum levels of IFN-γ and IL-2

In general, SE-vaccinated chickens in both age groups showed significantly higher serum levels of IFN-γ and IL-2 following primary and secondary immunizations compared with age-matched controls (Fig. 5). This effect was more apparent in serum IFN-γ than serum IL-2 levels. Two peaks in IFN-γ production were observed at 7–11 days ppi and 3–6 days psi in both age groups whereas serum IL-2 was increased over controls only at 11–14 days ppi (8 mo) and day 11 ppi (4 wk).

3.4. Splenic lymphocyte proliferative responses to SE OMP or flagella

The second set of experiments were designed to determine the effects of vaccination with the Poulvac® killed SE vaccine on mitogen- or antigen-induced splenocyte proliferation and serum nitric oxide (NO) and cytokine levels. In the control group, no significant change in percentages of $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ cells were

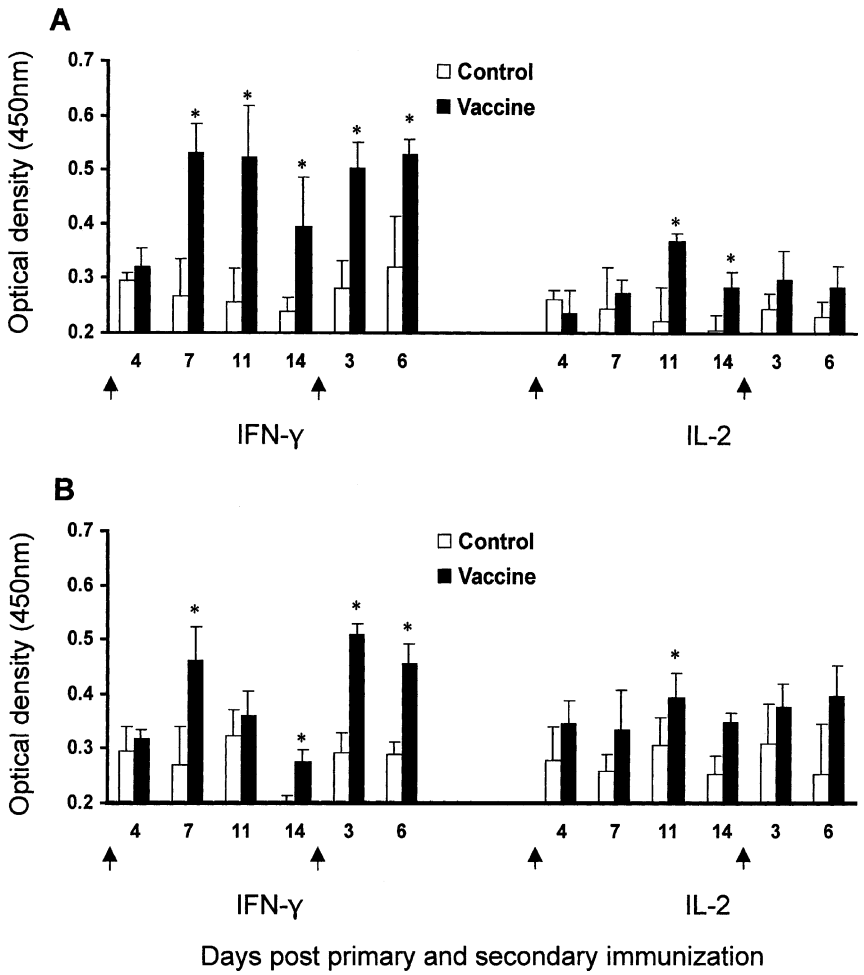

Fig. 5. Serum IFN-γ and IL-2 levels from the chickens vaccinated at 8 mo (A) or 4 wk (B) of age. Arrows indicate days when the first (day 0) and second (day 15) vaccinations were given. At 4, 7, 11, and 14 days ppi, and 3 and 6 days psi, serum samples were collected from vaccinated and control groups and IFN-γ and IL-2 levels measured by ELISA. Each bar represents the mean ± SD of three birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$).

observed throughout the experiment (Table 1). The vaccinated group also showed a similar pattern of T-cell subpopulations to the control group, except for TCRγδ$^+$ cells, which were significantly increased after stimulation with flagella in splenocytes obtained at 7 days ppi.

3.5. NO production by spleen cells stimulated with ConA or SE antigens

At 4 days ppi, spleen cells from SE-vaccinated chickens showed significantly higher NO production compared with non-vaccinated control chickens following stimulation

Table 1
T-cell subpopulation in spleen cells proliferating upon stimulation with SE flagella

	Control chickens								Vaccinated chickens							
	No stimulation (medium alone)				Stimulation with flagella				No stimulation (medium alone)				Stimulation with flagella			
	CD4[a]	CD8	TCRαβ	TCRγδ	CD4	CD8	TCRαβ	TCRγδ	CD4	CD8	TCRαβ	TCRγδ	CD4	CD8	TCRαβ	TCRγδ
4 days ppi	32.45	50.00	48.19	22.68	35.80	42.40	34.93	20.89	26.81	41.41	39.08	25.19	24.71	43.07	45.84	19.67
7 days ppi	22.03	45.63	47.93	13.50	22.27	45.62	50.03	18.38	26.79	47.99	50.81	15.80	22.92	47.30	45.89	50.88*
11 days ppi	36.86	48.38	53.06	24.07	23.75	34.85	46.26	21.49	28.85	40.61	56.32	14.37	23.00	42.40	46.72	19.61
14 days ppi	32.34	45.49	50.94	11.37	14.74	38.56	47.78	22.11	35.22	32.23	43.86	12.92	22.81	32.05	39.41	22.91
3 days psi	28.69	43.27	40.50	14.69	13.94	40.40	38.18	21.63	30.40	37.84	48.32	17.10	25.20	35.89	44.04	21.89
6 days psi	27.58	39.83	37.39	17.90	16.21	41.19	40.03	20.57	32.63	37.05	46.98	17.15	24.46	40.40	43.03	21.35

Data were expressed as mean percentages of T-cell subpopulations in splenocytes from 6 chickens/group.

*Significant difference between the treatments in the vaccinated chickens ($P < 0.05$), and between the groups at the same time point ($P < 0.05$).

[a] Percentages of T-cell subpopulations recognized by mAbs against each marker were calculated with respect to those stained by K55 mAb (a pan lymphocyte marker).

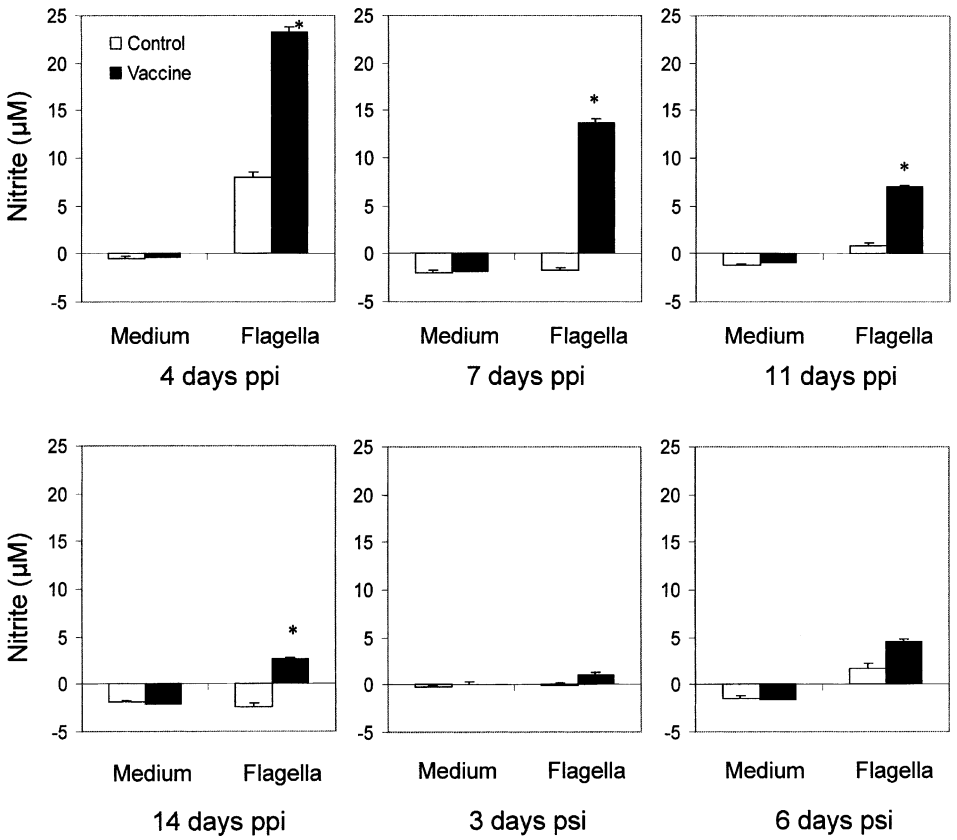

Fig. 6. Flagella-stimulated NO production by splenocytes from chickens vaccinated at 3 wk of age. Each bar represents the mean ± SD of six birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$). in supernatants from splenocytes cultured with or without flagella for 48 h were measured using Griess reaction. The nitrite concentration was determined using a standard curve generated with sodium nitrite (μM). Each bar represents the mean ± SD of six birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$).

with flagella (Fig. 6). However, NO production gradually decreased such that by 3 and 6 days psi, no significant differences were noted between vaccinated and non-vaccinated birds.

3.6. Levels of IFN-γ, IL-1, IL-6, and IL-8 in sera

SE-vaccinated chickens showed significantly increased serum levels of IFN-γ (Fig. 7A), IL-1 (Fig. 7B), IL-6 (Fig. 7C), and IL-8 (Fig. 7D) at 7 days ppi ($P < 0.05$). However, no differences were noted at any of the other time points examined.

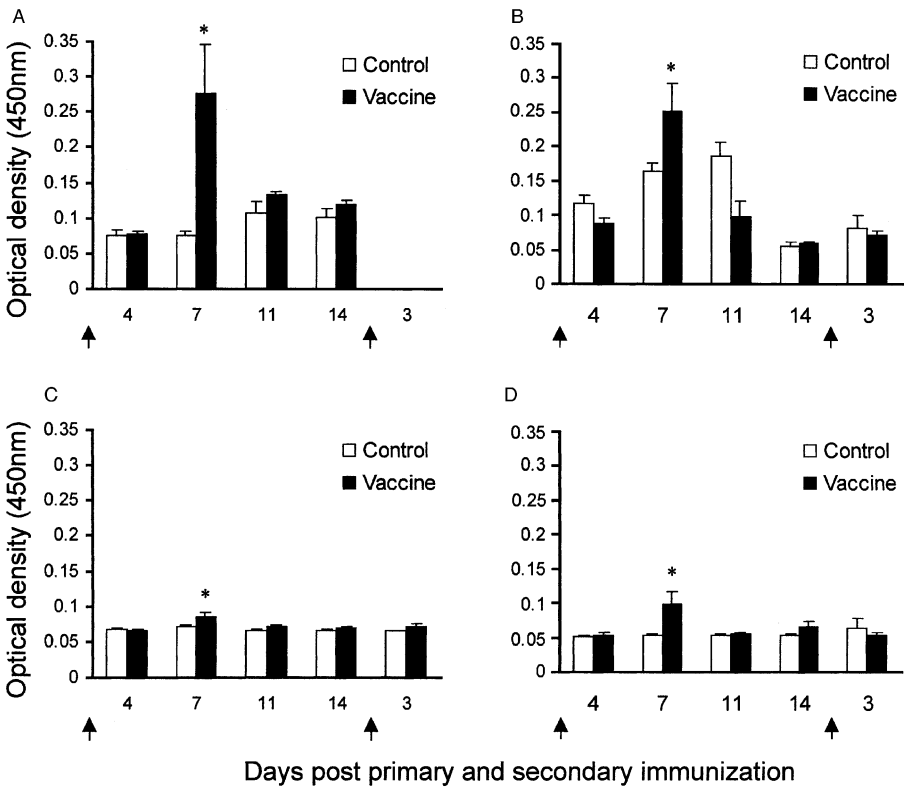

Fig. 7. Serum IFN-γ (A), IL-1 (B), IL-6 (C), and IL-8 (D) levels from chickens vaccinated at 3 wk of age. Arrows indicate primary (day 0) and secondary (day 15) vaccinations. At 4, 7, 11, and 14 days ppi and 3 and 6 days psi, serum samples were collected and cytokine levels measured by ELISA. Each bar represents the mean ± SD of six birds. Asterisks represent significant differences between the vaccinated and control groups ($P < 0.05$).

4. Discussion

The first set of experiments comparing the effects of host age on antigen-specific splenocyte proliferation demonstrated that SE LPS- and SE flagella-induced proliferation were significantly higher in 4-wk-old chickens compared with 8-mo-old chickens following vaccination with a killed commercial SE vaccine. Similarly, the trends in IFN-γ and IL-2 production by splenocytes were generally higher in younger chickens compared with older animals. These results provide an immunological basis for previous observations on the effect of host age on SE infection. For example, several studies on the effect of age differences in the host resistance to salmonellosis have demonstrated that the immature immune system of chickens shortly after hatching is generally unable to clear enteric SE infections. Thus, SE-induced mortality was much higher in 1-day-old chicks compared with 3-wk-old birds [32]. In addition, adult chickens at the age of about 20–37 wk were less susceptible to SE infection than at about 60 wk of age [15,18].

LPS is a polyclonal B-cell mitogen which accounts for our observations that IFN-γ and IL-2 secretion by spleen cells from 4-wk-old SE-vaccinated birds was not elicited upon stimulation with LPS. Flagella, on the other hand, was the most immunogenic component to induce the antigen-specific proliferative response and cytokine secretion of splenocytes in 4-wk-old chicks. Previous studies have shown that *Salmonella* flagella efficiently captured antigen-specific antibodies against *Salmonella* with high sensitivity [33–36] although the nature of cells responding to the flagellar antigen is not known. Hassan and Curtiss [23] found that chickens orally immunized with avirulent live *Salmonella typhimurium* showed an increased delayed type hypersensitivity (DTH) response to the flagellar antigen. In mice, McSorley et al. [37] showed that $CD4^+$ T lymphocytes are the major cells producing IFN-γ in response to flagellar antigen. We can therefore assume that T lymphocytes are responsible for the proliferative response by splenocytes observed in our study.

Furthermore, splenic $TCR\gamma\delta^+$ cells showed a significant increase following in vitro stimulation with SE flagella antigen (Table 1). $TCR\gamma\delta^+$ cells may play important roles in mucosal defense against chicken intestinal pathogens [38]. A recent study [39] demonstrated significant elevation of $CD8^+TCR\gamma\delta^+$ cells in blood, ceca, and spleen after oral administration of attenuated or non-attenuated *S. typhimurium* strains. While the majority of the $TCR\gamma\delta^+$ cells might express CD8 antigen in the chicken spleen [40], the minimal changes in $CD4^+$ or $CD8^+$ cells between the vaccinated and the control groups indicate that $TCR\gamma\delta^+$ cells probably do not express either cell surface antigen. Additional studies using multi-color staining procedures to assess $TCR\gamma\delta^+$/CD8-double positive cells are needed to address this question. Since limited information is available on the functional role of $TCR\gamma\delta^+$ cells in immunity to *Salmonella* infection in birds or mammals, the mechanisms by which $TCR\gamma\delta^+$ cells contribute to protection against *Salmonella* deserve further elucidation.

NO is generally produced by host macrophages in response to invading *Salmonella*. We observed that the killed SE vaccine induced increased NO production in splenocytes stimulated with SE flagella. Similarly, flagella protein from *S. typhi* was also shown to be a powerful monocyte activator [41]. Although we did not study in great detail the role of NO in SE immunity, the maleficent effect of macrophage- or antigen-presenting cell-derived NO on antigen-specific T cell proliferation is documented in mammalian systems [42,43]. The lack of a lymphocyte proliferative response to ConA, a polyclonal T-cell mitogen, after 7 days ppi in both age groups of the present study may be related to immunosuppression [23,24], possibly NO-induced [44]. Whereas the meaning of this inhibitory effect on host protection against *Salmonella* has been controversial, this effect is considered to occur during the early stages of bacterial infection via the innate immune response pathway and involving IL-12, NK cells, and IFN-γ [43]. In our study, T-cell-mediated immunity (i.e. proliferation of $TCR\gamma\delta^+$ cells specific to the *Salmonella* antigens in vitro and the corresponding peak of IFN-γ production in vivo) might be induced as the innate response (i.e. increased NO production) begins to decrease at 7 days ppi. Interestingly, the studies of Arnold and Holt [24] suggested that immunosuppression induced by *Salmonella* occurred at 1–2 wk post-infection.

IFN-γ, a major Th1 cytokine produced by T helper ($CD4^+$) cells and NK cells, has been demonstrated to play an important role in protection against *Salmonella* infection in avian

hosts [45–49]. The most likely mechanism by which IFN-γ exerts its functions in host defense against *Salmonella* is the activation of macrophages to induce NO production [50,51] and MHC class II expression [52]. The increase in serum IFN-γ and IL-2, also a Th1 cytokine with T-cell proliferative activity [53], reported in the present study strongly suggests induction of the Th1 axis in response to SE vaccination.

In mice, it is well accepted that pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α are produced by macrophages to induce acute phase proteins following pathogen challenge [54,55]. The functional role of IL-1 and IL-6 in protection of hosts from *Salmonella* is not clear because these cytokines have not been explored to the same extent as IFN-γ. However, IL-1 and IL-6 seem to have particular effects on the host response to *Salmonella* in mice [56,57]. Recent studies found that IL-1 and IFN-γ mRNA expression were enhanced by challenge of chickens with *S. typhimurium*-derived LPS [58]. Xie et al. [59] also observed elevated IL-6 production upon i.v. administration of chickens with *S. typhimurium*-derived LPS. These results were observed in the early phase of *Salmonella* infection or LPS injection whereas our study showed the high responses of IL-1, IL-6, and IFN-γ at 7 days post immunization (Fig. 1). This difference might be due to the chickens' age and/or genetics, LPS types, or route of immunization.

IL-8 is known as a chemokine that recruits polymorphonuclear leukocytes to inflammatory sites. In mice, intestinal epithelial cells have been demonstrated to produce IL-8 in response to invasion by *Salmonella* [60–62]. In chickens, it was demonstrated recently that heterophils, the avian counterpart of mammalian neutrophils, are recruited to the primary site of *Salmonella* infection by IL-8 [63]. Because our study used the killed vaccine by parenteral administration, we expect that macrophages rather than intestinal epithelial cells might be responsible for the observed response in IL-8 levels. This is supported by others [64] who reported that the chicken macrophage inflammatory protein which is encoded by the 9E3/CEF4 DNA shows high homology to mammalian IL-8 and is abundantly expressed by activated peripheral blood monocytes.

In general, killed vaccines have not been considered to be as efficient as live attenuated vaccines in eliciting protective immunity to *Salmonella* [19,65–67]. While killed vaccines generate an acceptable level of antibody production, they are poor inducers of T-cell-mediated immunity [68–70]. It must be noted, however, that our study demonstrated T-cell-mediated immune responses manifested by IFN-γ production and antigen-specific proliferation of TCRγδ^{+} cells after vaccination with the killed SE bacterin although the age of host affected the degree of this cellular immunity. Moreover, the increased level of IL-1, IL-6, and IL-8 and in vitro NO production indicates that the host inflammatory and pro-inflammatory responses were clearly augmented by the killed vaccine.

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